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Distinguishing the externally similar imagines of *Tiracola plagiata* and *T. aureata* whose forewing lengths were shown to overlap (Lepidoptera, Noctuidae)

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Abstract Two species of the genus *Tiracola* Moore known from Japan, *T. plagiata* and *T. aureata*, have very similar wing markings and very variable coloration of the forewings. They have been considered to be distinguishable easily from each other by the lengths of the forewings. Usually *T. aureata* has been believed to be larger than *T. plagiata*. However, by measurement of the forewings, we have found that some specimens of *T. plagiata* are larger than *T. aureata*. We have therefore developed discrimination methods for them using the female genitalia and standard DNA barcoding. As a result, we could distinguish them by the characteristics of the antrum of the female genitalia. Furthermore, we could distinguish them successfully using standard DNA barcoding (*COI*).

Key words DNA barcoding, female genitalia, mt*COI*, *Tiracola aureata*, *Tiracola plagiata*.

Introduction

The genus *Tiracola* Moore, 1881 comprises 13 species worldwide (Poole, 1989; Holloway, 1989): two species, *T. plagiata* (Walker) and *T. aureata* Holloway, have been recorded from Japan (Sugi, 2001; Yoshimatsu, 2011). The larva of *T. plagiata* seems to be highly polyphagous and is known as a pest of *Theobroma cacao* (Cacao), *Hevea* spp. (Para rubber tree) and *Pinus merkusii* (Merkus pine) in Indonesia (Kalshoven and Laan, 1981) and *Citrus* spp. in Taiwan (Sugi, 1982). But, as mentioned by Holloway (1989), host records of *T. plagiata* should be carefully checked because they may include those of *T. aureata* also.

Both species have very similar wing markings and very variable coloration of the forewings (Figs 1-4), but they are easily distinguishable by the male genitalia as illustrated by Holloway (1989). Additionally, it was thought to be easy to distinguish them because *T. plagiata* had been believed to be usually smaller than *T. aureata* as mentioned by Sugi (2001). However, from our detailed study we discover that while *T. plagiata* is generally significantly smaller than *T. aureata*, the lengths of their forewings overlap in some specimens.

Therefore, to establish an exact identification method for both species, we investigate in this paper the morphological differences between their female genitalia and we also consider the possibility of distinction between them by standard DNA barcoding.

Materials and methods

Measurement of forewings

For the measurement of lengths of forewings of the specimens, we used a total of 37 adults from Japan, Vietnam, Laos, Taiwan, Philippines, Malaysia, Indonesia and India. Their forewing lengths were measured by digital scales (MITUTOYO, ABS Digimatic Caliper CD-15CX) individually. In addition, we also examined the significant difference of length between forewings of both species using the Mann-Whitney *U* test.

As a first step all individuals including males and females were dissected and we could identify all males easily. Secondly, we examined several smaller female specimens (≦20.9 mm) which we regarded as incontrovertibly *T. plagiata* and from this obtained an understanding of its female genitalia. Similarly, we also examined several larger female specimens (≧26.0 mm) which we considered to be *T. aureata* and hence obtained an understanding of its female genitalia. In this way, we established the characteristics of the female genitalia of both species. On this basis we could identify the medium sized female individuals (>20.9 mm and ≦25.9 mm) by the female genitalia.

DNA experiments

A total of ten adult specimens collected from Vietnam in 2012 and 2013 at Light Trap were used for experiments. All legs on the right side of the body of all specimens collected were removed and one leg was put into 99.5% ethanol for subsequent DNA extraction; DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden,

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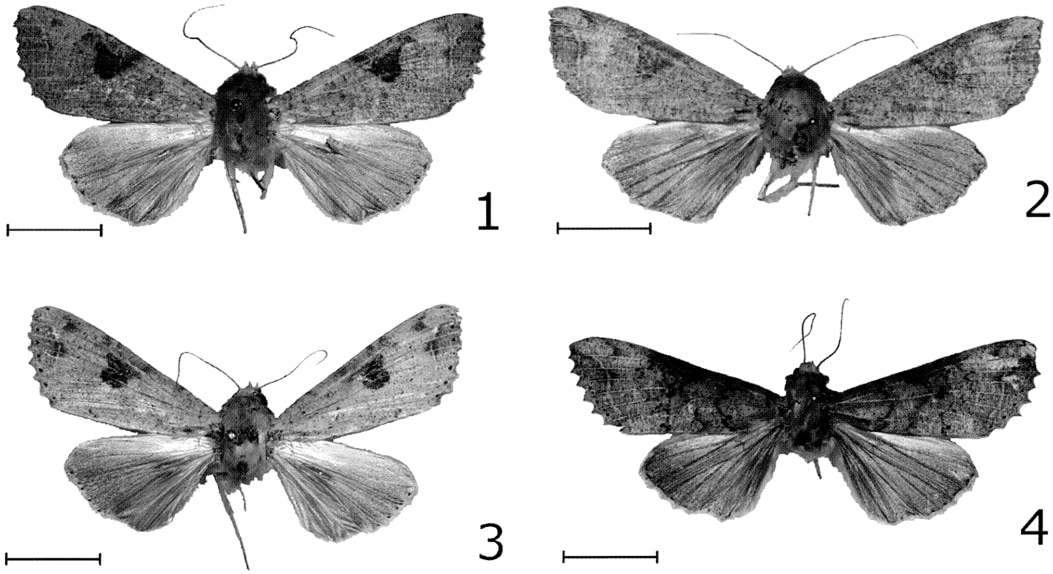

Figs 1–4. *Tiracola* spp. (Scale bars = 10 mm; LF = Lengths of forewings)
1: *T. plagiata*, ♂ (LF 23.9 mm). 2: Ditto, ♀ (LF 24.5 mm). 3: *T. aureata*, ♂ (LF 22.2 mm).
4: Ditto, ♀ (LF 22.3 mm).

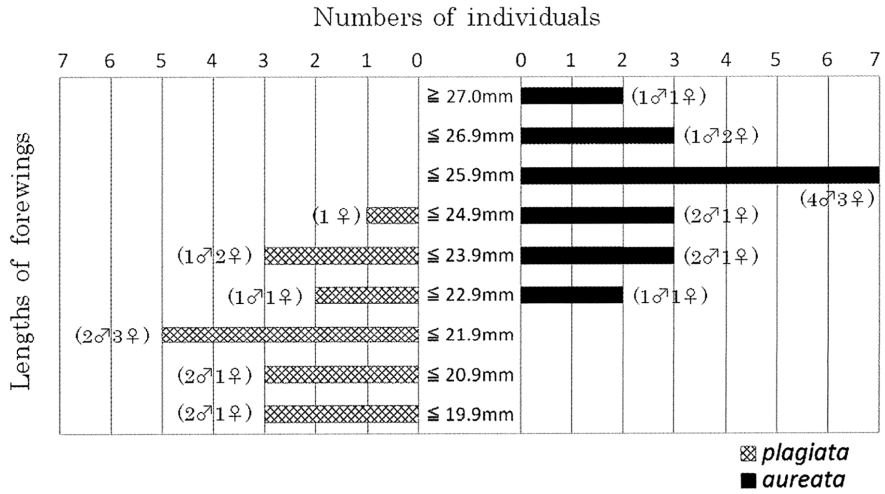

Fig. 5. Lengths of forewings and numbers of individuals of *T. plagiata* and *T. aureata* falling within each band. Males and females are distinguished and their numbers are also shown.

Table. 1. Numbers of individuals examined and lengths of forewings of *T. plagiata* and *T. aureata*.

Species name	No. of individuals	Lengths of forewings (mm, Mean±SE)	*Z*	*p*
T. plagiata	17	21.6±1.6	4.604	$p<0.0001$
T. aureata	20	25.1±1.5		

*Lengths of forewings of two species are significantly different by Mann-Whitney *U* test.

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Germany). Usually the remaining two legs were kept in a freezer at –30 °C using a 99.5 % ethanol soaked tube for further study of other regions of DNA. The leg was homogenized in 200 µl ATL-Buffer with 20 µl proteinase K and incubated at 55 °C for one hour. After incubation, total genomic DNA was extracted following the manufacture's instructions. A DNA fragment of the mitochondrial cytochrome oxidase subunit I (*COI*) gene was amplified using the polymerase chain reaction (PCR) with the primers LCO1490: (5'-GGT CAACAAATCATAAA GATTGG-3') and H CO2198: (5'-TAAACTTCAGGGTGA CCAAAAAA TCA-3') (Folmer *et al.*, 1994). The template profile was as follows: 94.0 °C for 300 sec, 40 cycles at 94.0 °C for 30 sec, 47.0 °C (except for two examples; Accession Number AB827313 and AB827314 were done at 46 °C) for 30 sec, 72.0 °C for 60 sec; with final extension at 72.0 °C for 300 sec. PCR was preformed in a reaction volume of 40 µl using, 3.2 µl of dNTP Mixture, 4.0 µl of each primer (10 µmol/l), 4.0 µl of 10×EX Taq Buffer (Takara Bio, Tokyo, Japan), 0.4 µl of TaKaRa EX Taq (Takara Bio), 2.0 µl of template DNA, and 22.4 µl of SDW. We asked Takara Bio Inc. to do the analysis of nucleotide sequences by direct sequencing using a Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and an ABI 3730-XL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

We used MEGA5 software (Tamura *et al.*, 2011) to align nucleotide sequencing and to construct a neighbor-joining (NJ; Saitou and Nei, 1987) tree using p-distance. To estimate the confidence probability for each interior branch, the bootstrap method (Felsenstein, 1985) was performed with 1,000 replications. We used DNAsp v5 (Librado and Rozas, 2009) to search for sequence divergence between the two species.

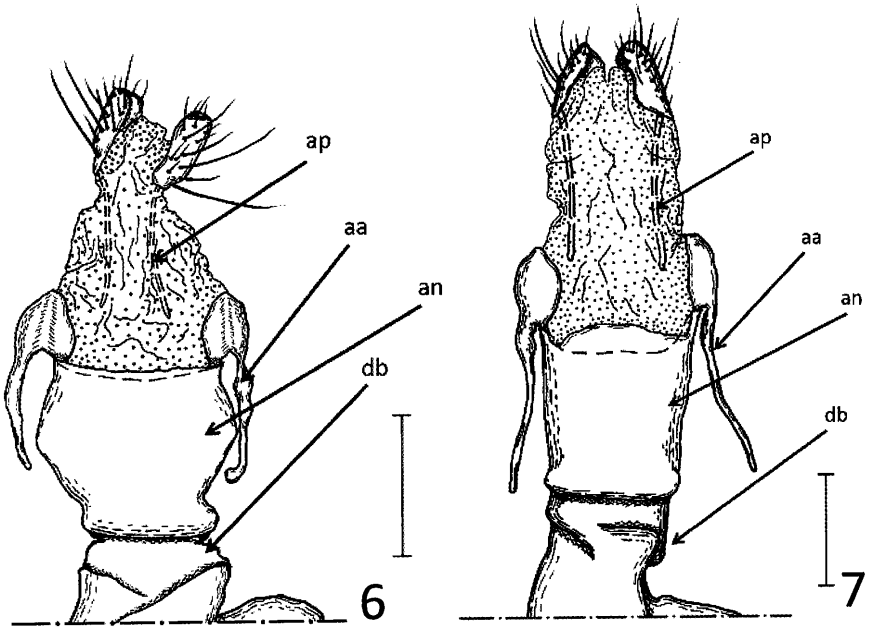
Specimens examined

Tiracola plagiata (Walker)

[Vietnam] 1♀, Dak Lak Prov., Krong Bong District, Chu Yang Sin National Park, alt. 733m, 12. iii. 2013, T. Mita *et al.* (Accession No; AB827314); [Philippines] 1♂, Negros, 1991; [Japan] 1♀, Okinawa-Is., 21. ii. 2000, S. Tominaga; 1♂, ditto, 28. xi. 1999, S. Tominaga; 1♂, ditto, 13. xi. 1999, S. Tominaga; 1♀, ditto, 17. xi. 1999, S. Tominaga; 1♀, Okuni-Rindou, Kunigami-vill., Okinawa., 27. v. 2000, A. Sasaki; 1♀, Kin-town, Okinawa, 28. v. 2000, A. Sasaki; 1♀, ditto, 8. viii. 1995, K. Kudo; 1♀, Tengan riv., Okinawa, 22. vii. 1998, K. Kudo; 1♂, Gushikawa, Okinawa, 14. vi. 1997, K. Kudo; 1♀, Mt. Banna, Ishigaki-jima, Okinawa, 3. vi. 1973, Y. Kurosawa; 1♀, Chichi-jima, Ogasawara, Tokyo, 19. v. 1995, K. Takeuchi; [Malaysia] 2♂, Taiping, viii. 1973; 1♂, ditto, vii. 1973; [Indonesia] 1♂, Berastagi, N. Sumatra, 27. iv - 4. v. 1988, S. & A. Saito.

Tiracola aureata Holloway

[Vietnam] 4♂3♀, Lao Cai Prov., Tram Ton, Sapa, 1926m, 22-23, vi. 2012, S. Yoshimatsu & D. Watabiki (Accession No: AB827305-AB827306, AB827308- AB827312); 1♀,



Figs 6–7. Distal half of female genitalia of *T. plagiata* (Fig. 6) and *T. aureata* (Fig. 7) in ventral view. Scale bars = 1 mm. Following abbreviations as in Komai (2011) are used. ap; apophysis posterioris, aa; apophysis anterioris, an; antrum, db; ductus bursae.

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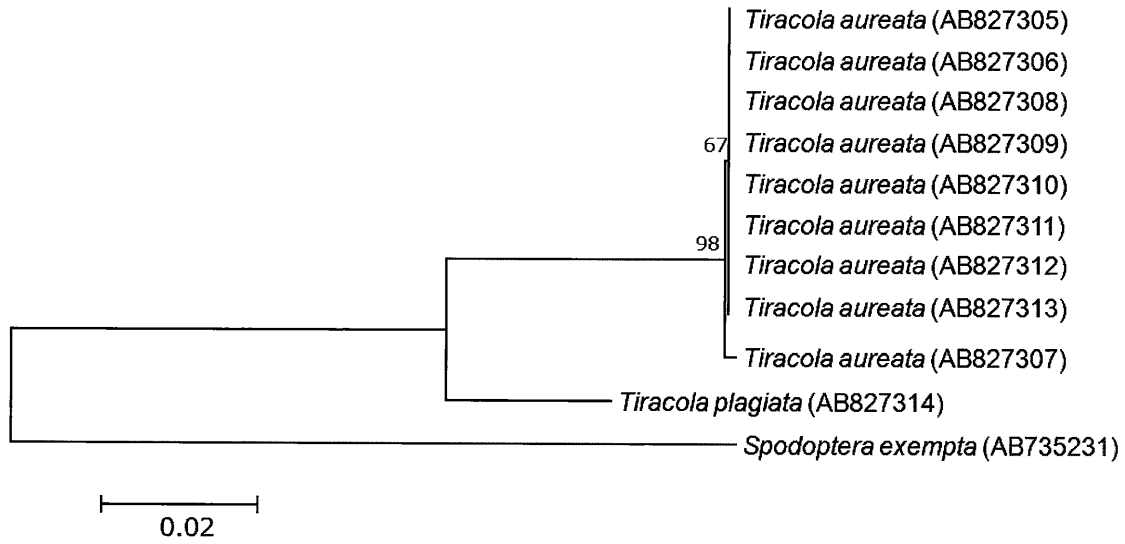

Fig. 8. Neighbor-joining tree of *Tiracola* spp. based on 616 bp of the mitochondrial *COI* gene. *Spodoptera exempta* quoted from Genbank was used as an outgroup species. The bootstrap method was performed with 1,000 replications.

Lao Cai Prov., Ta Phin, 1296 m, 25. vi. 2012, S. Yoshimatsu & D. Watabiki (Accession No: AB827307); 1♂, Dak Lak Prov., Krong Bong District, Chu Yang Sin National Park, alt. 733 m, 13. iii. 2013, T. Mita *et al.* (Accession No; AB827313); [Laos] 1♀, Xieng Khouang Prov., Phou Samsoum, alt. 1940m, S. Yoshimatsu & D. Watabiki; [Indonesia] 3♂, Bali, west, ix. 1994; [Taiwan] 1♂1♀, Chiayi, 12–13. vii. 1964, H. Inoue; [Malaysia] 2♀ Taiping, vii. 1987; [India] 1♂, Darjeeling, v. 1975, T. Hasegawa; 1♂1♀, Sabah, Mt. Kinabalu, 16–21, iii. 1977, N. Kashiwai.

Voucher specimens are deposited in the Natural Resources Inventory Center, National Institute for Agro-Environmental Sciences (NIAES).

Results and Discussion

The results of measurement of the length of forewings are shown in Fig. 5. The results apparently show that there is no significant difference between the length of forewings of male and female. However, a significant difference was observed between the respective lengths of the forewings of each species (Table 1).

The male genitalia of *T. aureata* were illustrated by Holloway (1989) and Kononenko and Han (2007). However, the female genitalia of *T. plagiata* have not been illustrated and no distinguishable characteristics of the female genitalia of these two species have been given until now. Therefore, here we show discrimination methods for both species using the female genitalia. The most reliable character to distinguish them is that the antrum of *T. plagiata* is wide and protrudes to produce an angle in the middle of both sides (Fig. 6), while that of *T. aureata* is narrow and never protruding at the sides (Fig. 7).

Additionally, the male genitalia of both species are obviously different. The most reliable distinguishing feature is mentioned as follows by Holloway (1989) "In *aureata* the cucullus is triangular, the ventral angle (with spur or digitus) approximately level with the dorsal one, whereas in *plagiata* the dorsal angle is more rounded and strongly produced relative to the ventral one (though this is less pronounced in Pacific populations of *plagiata*) etc.".

In addition, we also developed a discrimination method using standard DNA barcoding. As a result, we could distinguish them successfully by this method also. We registered their nucleotide sequences in the DNA Data Bank of Japan (DDBJ) and we show the accession numbers of those registered specimens in Fig. 8. There was 5.1% sequence divergence between the two species. This percentage of sequence divergence is comparable to the average numerical value, 5.6% of six Japanese *Spodoptera* pest species as mentioned in Watabiki *et al.* (2013).

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Kononenko, V. S. and H. L. Han, 2007. In Park, K. T. (ed.), Atlas genitalia of the Noctuidae in Korea (Lepidoptera), Insects of Korea (11), 464pp., Jungheng Publishing Co., Seoul.

Librado P. and J. Rozas, 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.

Poole R. W., 1989. Lepidopterorum Catalogus (New Series) Fascicle 118 Noctuidae Part1–3, 1,314 pp., E. J. Brill.

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摘　要

前翅長が重複することが判明した酷似種ノコバヨトウ (*Tiracola plagiata*) とオオノコバヨトウ (*Tiracola aureata*) を識別する（綿引大祐・吉松慎一）

Tiracola 属（ヤガ科：ヨトウガ亜科）には世界から13種が知られている．日本からは東南アジアの広域分布種でもあるノコバヨトウ *T. plagiata* (Walker) とオオノコバヨトウ *T. aureata* Holloway の2種が記録されている (Sugi, 2001; Yoshimatsu, 2011)．ノコバヨトウは非常に広食性の様で，インドネシアではカカオ *Theobroma cacao* やパラゴムノキ属 *Hevea* 等の害虫としての記録があり（Kalshoven and Laan, 1981)，台湾からは柑橘類の害虫として記録されているが (Sugi, 1982)，両種は近年まで混同されてきたために (Holloway, 1989)，寄主植物については今後の再検討が必要である．

両種の翅の斑紋は類似するのに加えて，色彩は共に非常に変異に富み (Figs 1–4)，外見的に両種を識別することは非常に難しいが，オオノコバヨトウはノコバヨトウより常に大きいことから両種の識別は可能だとされてきた (Sugi, 2001)．ところが，筆者らが手元にある両種の標本の雌雄交尾器と前翅長を詳細に比較検討したところ，両種の前翅長には有意差があったものの (Table 1)，ノコバヨトウの中にはオオノコバヨトウより前翅長の長いものや同等の前翅長を持つものが存在することが判明した (Fig. 5)．両種の雄交尾器は，Holloway (1989) によって明瞭な識別点が図示されているが，両種の雌交尾器に関してはこれまで検討されてこなかった．そこで筆者らが両種の雌交尾器を詳細に観察したところ，antrum はノコバヨトウでは幅広く，側方に三角形状に突出するのに対し，オオノコバヨトウでは幅が狭く，突出しないことで両種は簡単に識別できることが今回新たに判明した (Figs 6, 7)．また，今回ベトナム産の両種の新鮮な標本が得られたことから，ミトコンドリア *COI* の一部領域の塩基配列を解読し，標準的な DNA バーコーディングによる両種の識別を試みたところ，有効であることが示された．さらに，Watabiki *et al.* (2013) において著名な害虫ハスモンヨトウ等を含む日本産ヤガ科 *Spodoptera* 属6種の DNA バーコーディング領域の種間の平均の塩基置換率は5.6%であることが示されているが，本研究においてノコバヨトウとオオノコバヨトウの種間の塩基置換率を算出したところおよそ5.1%であり，日本産 *Spodoptera* 属6種の種間の平均の塩基置換率と類似の値を示したので，参考までにここで触れておく (Fig. 8)．

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